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Molecular characterization by MLVA of *Coxiella burnetii* strains infecting dairy cows and goats of north-eastern Italy

Letizia Ceglie [a,*], Eulalia Guerrini [a], Erika Rampazzo [a], Antonio Barberio [b], Jeroen J.H.C. Tilburg [c], Ferry Hagen [c], Laura Lucchese [a], Federica Zuliani [a], Stefano Marangon [a], Alda Natale [a]

[a] *Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro PD, Italy*
[b] *Istituto Zooprofilattico Sperimentale delle Venezie, Viale Fiume 78, 36100 Vicenza, Italy*
[c] *Canisius-Wilhelmina Hospital, Department of Medical Microbiology and Infectious Diseases, Nijmegen, The Netherlands*

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Abstract

Q fever is a worldwide zoonotic disease caused by *Coxiella burnetii (C. burnetii),* an obligate intracellular bacterium. In ruminants, shedding into the environment mainly occurs during parturition or abortion, but the bacterium is shed also in milk, vaginal mucus, stools and urine. In Italy few surveys have been conducted and reported seroprevalence values ranged between 10% and 60%, even if few human cases have been described. Genotyping of bacteria is crucial for enhancing diagnostic methods and for epidemiological surveillance. The objective of this study was to investigate genotypic differences of *C. burnetii* genotypes directly in 34 samples, collected during a 3-years survey among 11 dairy cattle and 11 goat farms in the north-eastern part of Italy using a 6-locus multiple loci variable number of tandem repeat analysis (MLVA) method. The samples analysed included 13 bulk tank milk (BTM), 6 individual milk, 11 vaginal swabs and 4 foetal spleens. MLVA-type 2 was determined as the most prevalent in cattle in this study. *C. burnetii* strains circulating in the studied cattle population are very similar to genotypes previously described, while genotypes from goats showed an important variability. Further investigation are needed to understand the reason of this pattern.

Keywords: *Coxiella burnetii*; Dairy farms; Typing methods; Molecular epidemiology; Italy

1. Introduction

Q fever is an infective and contagious disease caused by *Coxiella burnetii*, an obligate intracellular bacterium [1,2]. After the Dutch outbreak in the years 2007–2011 with over 3500 human cases, epidemiological studies on Q fever have increased and the need to understand the relationships between human and animal outbreaks became imperious [3,4]. The infection in ruminants is frequently subclinical, but late abortions, stillbirths and reproductive disorders can occur [5]. Shedding of *C. burnetii* into the environment mainly occurs during parturition or abortion, but the bacterium is also shed in milk, vaginal mucus, urine and stools. Identifying the source of an infection is complex because different reservoir and vector diversity are concerned. Therefore molecular characterisation of strains is crucial to compare genotypes isolated from different animal species, to trace outbreaks and to assess relationships between genotype and virulence of the strains with a special regard to public health [3,6]. Availability of complete genome sequences has allowed to apply to this

* Corresponding author.
E-mail addresses: lceglie@izsvenezie.it (L. Ceglie), eguerrini@izsvenezie.it (E. Guerrini), erampazzo@izsvenezie.it (E. Rampazzo), abarberio@izsvenezie.it (A. Barberio), j.tilburg@cwz.nl (J.J.H.C. Tilburg), f.hagen@cwz.nl (F. Hagen), llucchese@izsvenezie.it (L. Lucchese), fzuliani@izsvenezie.it (F. Zuliani), smarangon@izsvenezie.it (S. Marangon), anatale@izsvenezie.it (A. Natale).

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bacterium many highly discriminatory methods, mainly based on molecular techniques like multispacer sequence typing (MST), IS1111-element positioning, infrequent restriction site-PCR (IRS-PCR), multiple loci variable number of tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP) [7,8]. Recently, researchers from different countries have applied the MLVA method to *C. burnetii* strains because of its quite high discriminatory power and its applicability either to isolated bacterial strains or directly to DNA extracted from clinical samples [9,10]. French researchers identified within the *C. burnetii* genome a total of 17 different minisatellite and microsatellite markers to be used in MLVA genotyping in 2 successive panels [9]. A third panel was implemented during the Dutch *C. burnetii* outbreak, involving 6 microsatellite markers [11,12]. The objective of our study was to investigate differences of the collected *C. burnetii* genotypes with this 6-locus MLVA panel [11,12] and to establish predominant MLVA-types in cattle and goats in the north-eastern part of Italy.

2. Materials and methods

2.1. Animal sampling and specimen processing before MLVA typing

The prevalence of Q Fever infection in dairy herds and the efficacy of a diagnostic approach based on *C. burnetii* detection in bulk tank milk by a real-time PCR were previously evaluated on dairy cattle in the north-eastern part of Italy [13,14]. A total of 11 cattle and 11 goat infected farms were selected for sampling between 2010 and 2013. Sampling was performed for 3 times, every two months in the designated farms and included vaginal swabs, abortive specimens (foetal spleen and abomasum), stools and individual milk samples. All collected samples were screened for the presence of *C. burnetii* by a real-time PCR (rPCR). DNA from milk, stools, organs and vaginal swabs was extracted by using the Qiamp DNA mini kit (QIAGEN SPA, Milan, Italy) and detected by means of the commercial real-time PCR kit (ADIAVET COX REALTIME−Adiagène), targeting the IS1111a element, on a 7900HT Fast Real time PCR system (Applied Biosystems), according to the manufacturer's instructions. A control DNA (GAPDH), referred as 'internal control', was present in each reaction in order to validate each negative result. Results were generated with the 7900HT Fast System Software (Applied Biosystems).

2.2. Multiple locus variable number tandem repeats analysis

Among infected farms, positive samples with Ct values <32 were primarily selected for further investigations by MLVA typing. Thus, all 11 selected cattle farms were included in the typing analysis. Since 7 out of 11 goat farms met the above criterion, in order to equilibrate the number of infected farms to be further investigated, additional samples, belonging the remaining 4 goat farms, with Ct values ranging from 32 to 34 were also included in the MLVA typing [3]. With regard to specimen and period of sampling, whenever possible, different specimens of samples (13 BTM, 6 individual milk, 11 vaginal swabs and 4 foetal spleen) have been collected and tested at different times within the same farm from different animal, to eventually check the variability between the genotypes. In only one case individual milk was collected from the same cow twice, in 2 consecutive years (Fig. 1 − Farm designation C9 2011 and C9 2012). As all positive stool samples showed Ct values greater than 34, the probability to succeed in typing was very low, therefore none of them were selected for typing.

The Dutch 6-locus MLVA panel was directly applied to the isolated DNA from 34 samples, using 2 groups of microsatellite markers: 3 hexanucleotide repeats markers (Ms27, Ms28 and Ms34), and 3 heptanucleotide repeats markers (Ms23, Ms24 and Ms33) [11,12].

For DNA reaction sequencing, each locus was amplified using one primer for each pair labelled at the 5′ terminus with a different fluorescent dye (FAM, HEX and Cy3), where FAM is 6-carboxyfluorescein, HEX is hexachlorofluorescein and Cy3 is fluorescent green cyanine, while the second primer was unlabelled [12].

The end-point PCR amplification was performed on a 9700 thermalcycler (Applied Biosystems) in a total volume of 20 µl containing 5 µl DNA extracted from each selected sample added to 15 µl of the reaction mix, enclosing 1X reaction buffer of the Fast Start Taq DNA Polymerase dNTP Pack kit (Roche Diagnostics, Monza, Italia), 1U of the Taq DNA Polymerase, 4 mM $MgCl_2$, 0.2 mM of a dNTP mix, and different final concentration each primer pair as follows 0.6 µM Ms23, 0.9 µM Ms33 and Ms24, 0.2 µM Ms34 and 0.5 µM Ms28 and Ms27. An initial denaturation/activation for 10′ at 95 °C was followed by 40 cycles of denaturation for 30″ at 95 °C, annealing for 30″at 60 °C, elongation for 60″ at 72 °C and a final extension step for 10′ at 72 °C. Amplicons were then run on acrylamide gels, stained with silver staining in order to verify amplification. DNA from Nine Mile RSA493 was used as reference strain and included in each run with all primers sets. After gel electrophoresis confirmation, the amplification product was diluted 1:10 with distilled water and 1.2 µl of each dilution was added to reaction mixture containing 10.5 µl distilled water and 0.3 µl of Gene Scan 500 size standard marker (Applied Biosystems). After denaturation for 3′ at 95 °C, samples were cooled on ice and a total volume of 10 µl was submitted to capillary electrophoresis on the Genetic Analyzer 3130 (Applied Biosystems). Each peak corresponded to a unique fluorophore, all amplicons of each group could be separated together in a ratio 1:3, in the POP_7 polymer of the capillary electrophoresis [3].

2.3. DNA sequencing and data analysis

After sequencing on the Genetic Analyzer® ABI 3130, the *C. burnetii* polymorphic VNTR sequences of the selected samples were analysed using GeneMapper 4.0 software and compared to the reference Nine Mile strain and the Q fever